DEVELOPMENT OF A FACIAL CREAM FOR ANTI-INFLAMMATORY CONTAINING HERBAL EXTRACTS

NINO DE MESA

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Cosmetic Sciences
2023

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By Nino De mesa

has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science in Cosmetic Sciences of Naresuan University

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ABSTRACT

When patients receive laser skin therapies, the irritation arises. In reaction to inflammation, and inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha), interleukins IL-1, & IL-8, and prostaglandin E2 (PGE_2). To treat skin inflammation issues, the simplest technique is to regularly apply a non-irritating facial cream. Plant extracts are increasingly being used for cosmetic and therapeutic reasons around the world. *Moringa oleifera*, *Derris scandens*, *Centhotheca lappacea*, and hemp seeds are exceptional plants that have shown anti-inflammatory effects in a variety of inflammation models.

The objectives of this study were, firstly, to investigate the anti-inflammatory activities of *Moringa oleifera* leaf extract, *Derris scandens* stem extract, *Centhotheca. lappacea* leaf extract, and hemp seed extract, and develop a facial cream containing these.

M. oleifera leaf extract were obtained by using 50% ethanol and *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seed extract were obtained by using 95% ethanol by maceration. Astragalin was the significant compound of interest found in *M. oleifera*. In *D. scandens*, the compound of interest was Lupalbigenin, while Coumaric acid was of interest in *C. lappacea*, and Linoleic acid in the hemp seeds. All plant extracts were evaluated for their toxicity to HaCat cells using an MTT Assay. Anti-inflmmatory activities were also measured. To

explore the levels of inflammatory inhibition demonstrated by the extracts, HaCat cells were pre-exposed to a 35 mJ/cm^2 dose of UVB to induce inflammation. Subsequently, concentrations of 1, 10, and 100 µg/ml of the various plant extracts were used to test their inhibition of Nitric Oxide (NO), IL-1a, IL-8, and PGE_2 in the pre-exposed HaCat cells.

The result for Nitric oxide (NO) test shows that all plant extract can inhibit nitic oxide production. However for IL-8 test, inhibition were greately seen in *C. lappacea leaf extracts* and *D. scandens* stem extract at concentration 10 µg/ml, markedly inhibit the production of interlukin IL-8. The largest decrease in Il-1α level was observed when the cells were treated with hemp seed seed extract at a concentration of 10 µg/ml. PGE_2 inhibition study suggested *M. oleifera* leaf extract and hemp seed extract at concetration 1 and 10 µg/ml demonstrated an inhibitory effect.

The dosage of the extracts used in our developed cream was based on the non-toxic concentration levels identified in the cytotoxicity tests that were conducted. The physical and chemical stability over time of the developed facial cream was also tested, and a preservative efficacy test was carried out on the facial cream which indicated that the preservative met the criteria of USP.

It can be concluded, therefore, that *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seed extract provide an inflammatory protection. Additionally, the biological activities of these extracts suggest that facial creams containing them have the potential for the treatment of skin inflammation.

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Nino De mesa

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CHAPTER I

INTRODUCTION

1. Statement of the problem

Many people in contemporary society consider health and beauty and cosmetic appearance to be important personal attributes requiring significant attention. This has led to significant advances in beauty treatments, skin creams and other cosmetic products and demand for these in a burgeoning market. Laser technology is an advancing technology being applied to skin and dermatological treatment (Gianfaldoni et al., 2017).

Light Amplification by Stimulated Emission of Radiation (LASER) skin resurfacing, also known as laser peeling or laser lasabrasion, is a minor skin rejuvenating surgical procedure that uses laser beams to lessen skin imperfections such as wrinkles, pigmentation spots, scars and blemishes. Laser beams eradicate the skin's outer layer, removing undesirable wrinkles, pigmented spots, scars, and blemishes. Simultaneously, the heat of the beams triggers the production of collagen fibers in the dermis. While the wound is healing, new skin will grow over it. The new skin will be smoother and firmer. A typical laser skin resurfacing uses a laser to ablate a range of problematic skin conditions. However, this treatment brings with it complex issues when the skin area being treated is large, requiring a long recuperation period. The risk of infection in this situation is high (WebMD Editorial Contributions, 2021).

Herbal extracts are currently being investigated all around the world for their effectiveness as ingredients in cosmetic products which have a huge market for such beauty products and skin and hair care (Chanchal et al., 2008). Some plants that are being used in the market for their anti-inflammatory effects are ginseng, jojoba, chamomile, licorice root, and fenugreek. Manufacturers are investing significantly large amounts of money to gain a profitable presence in this line of business.

There are many reports to be found in the literature on research and use of *Moringa oleifera* Lam. which has traditionally been used in traditional medicine for

the treatment of inflammation such as bronchitis, swelling, and pain (Anwar et al., 2007). Astragalin is a flavonoid that is one of the bioactive constituents found in *M. oleifera* leaves and its antioxidant capabilities and anti-inflammatory activities have been frequently reported (Han et al., 2004; Li et al., 2014; Luo et al., 2015; Soromou et al., 2012; Vongsak et al., 2014; Choi et al., 2013). One study reported that 50% ethanol extract of *M. oleifera* leaf significantly decreased the secretion of inflammatory mediators IL-1, IL-8 and PGE2 from UVB induced keratinocytes (Engsuwan et al., 2021). In recent years, there has been an increasing interest in products to relieve skin inflammation caused by ultraviolet (UV) radiation Type B (UVB). *M. oleifera* and Astragalin as their bioactive constituents have demonstrated efficacy in this regard.

Recently researchers have examined the effects of *Derris scandens* Benth as an anti-inflammatory, with free radical scavenging, antibacterial, antihypertensive, immunomodulatory, and anti-HIV properties. (Laupattarakasem et al., 2004; (Mahabusarakam et al., 2004; Sriwanthana et al., 2001) A wide range of biologically active chemical constituents were found in its stems and roots, which are chandalone, etunaagarone, nalanin, lonchocarpenin, osajin, robustic acid, scandenin, scandione, scandenone, scandinone, waragalone, wighteone, lonchocarpenin, and osajin (Thiangburanatham et al., 2013). The dried stems are used as an expectorant, antitussive, diuretic, and to treat arthritis, muscle and joint pains (Mahabusarakam et al., 2004). Previously, various portions of the *D. scandens* have been found to include coumarins, isoflavones, and glycosides as chemical components (Sreelatha et al., 2010; Mahabusarakam et al., 2004). There is a large body of published studies that describe the ethanolic extract of *D. scandens*. The *D. Scandens* extract was also able to reduce Erk1/2 activation and coercively stimulate the HT-29 cells to fatality by apoptosis, and mitotic cataclysm by the synthetization of gamma irradiation (Hematulin et al., 2014).

Centotheca lappacea has long been utilized in conventional remedies in Thailand and some southeast Asian countries for wound recuperation and assisting the restoration of vaginal muscle tissue and musculoskeletal tightening after giving birth (McClatchey et al., 1996; Pitiporn et al., 2014). The most commonly obtained secondary metabolites from the plants are phenolic compounds (Yeshanew et al.,

2020). Previous studies have reported that *C. lappacea* contains phenolic substances, such as catechin, gallate, rosmarinic acid, epigallocatechin, gallate and gallic acid (Thavatchai et al., 2017). Recent developments also show that *C. lappacea* consists of flavonoids, fatty acids, triterpenes and phytosterols, coumarins, stilbenes, hydrolysable and condensed tannins, lignans, and lignins (Blainski et al., 2013; Kamoltham et al., 2018). To study the radiation protection efficacy of *C. lappacea* extract in human endothelial cells, it was found that ethanol extract from *C. lappacea* has anti-radiation effects. While it is non-toxic and has no effect on the cell cycle of the EA.hy926 cells. Research has shown that C. *lappacea* extract is a radiation shielding agent that protects cells from being damaged (Meethang et al., 2020). Another study investigated the 4-coumaric acid for its protective effects against UVB-induced cell damage to a rabbit's cornea (Lodovici et al., 2009).

Hemp is a crop with many uses, and is a good natural supply of many elements that are specific to plants', particular their metabolisms (Flores-Sanchez et al., 2008). Its large variety of bioactive chemicals have a major role in its favorable effects on human health. (Martinez et al., 2020). It has a large variety of bioactive chemicals that play a major role in its favorable effects on human health (Martinez et al., 2020). Hemp seeds contain vitamins E, C, B1, B2, B3, and B6, and are approximately 25% protein, 30% carbohydrates, and 15% insoluble fiber, and includes carotene, phosphorus, potassium, magnesium, sulphur, calcium, iron and zinc. One of the best sources of necessary fatty acids for boosting the immune system are hemp seeds. It is also known to contain a favorable amount of gamma linoleic acid (GLA), while Hemp's GLA and vitamin D are helpful in the prevention and/or treatment of osteoporosis (Borhade et al., 2013).

Recent studies have demonstrated that linoleic acid extracted from *A. brasiliensis* reduces the production of NO and stops pro-inflammatory cytokines which include TNF-α, IL-6, IL-1, and NOS2 from being expressed in RAW 264.7 cells. This suggests that linoleic acid from *A. brasiliensis* can inhibit p50 and activate PPARα to lower the generation of NO and the inflammatory activity of RAW 264.7 cells (Saiki et al., 2017). *M. oleifera, D. Scandens* and *C. lappacea* are well known medicinal plants that are readily available in Asian countries.

Hemp was recently removed from the narcotics drug list in Thailand, enabling scientific research into its properties and compounds (Thai Food and Drug Administration, 2020). Given this, the objectives of this current research is to develop a facial cream as an anti-inflammatory reduction containing herbal extracts. The composition of the cream will include four main extracts to increase its efficiency and effectiveness in reducing inflammation thus reducing the potential for further skin damage and infection. As well, such a cream would be highly marketable and would add value to Thai herbs and be profitable for Thai manufacturers by promoting its use in cosmetics products. Significant outcomes of the research will also include guidelines for the production of the cream and quality control standards.

2. Purpose of the Study

1. To evaluate the anti-inflammatory effects of *M. oleifera, D. scandens, C. lappacea* and Hemp seeds extract.

2. To develop a product that has anti-inflammatory properties and contains herbal extracts.

3. Scope of the Study

In this study, the cytotoxic effect on keratinocyte cells by the ethanolic extracts of *M. oleifera, D. scandens, C. lappacea* and Hemp seeds, were chosen and elucidated the safety of the extracts. The inhibition of various cytokines were used to study the plant extracts' capacity to inhibit the infilammation. Additionally, further research was conducted on the most effective plant extract concentrations to ascertain its stability by examining its bioactivity, as well as the specific information of the plant extract.

4. Keywords

Moringa oleifera, Derris scandens, Centhotheca lappacea, Hemp seed, Anti-inflammatory

CHAPTER II

REVIEW OF LITERATURE AND RESEARCH

1. Introduction

Inflammation is the body's natural response to tissue damage, and it can cause redness, warmth, swelling, pain, and tenderness. Laser dermatological treatments are widely utilized to treat a variety of skin issues, including fine lines and wrinkles, hyperpigmentation, scars, and acne. While these treatments have the potential to be beneficial, they can also produce skin inflammation as a side effect.

Skin inflammation is a typical adverse effect of laser dermatological treatments, as the laser can cause skin damage, resulting in redness, swelling, and discomfort. Inflammation might last a few days or several months, depending on the type of laser treatment used and the individual's skin type and sensitivity. Several factors, including laser type, energy levels employed, planned depth of penetration, patient skin type, and post-treatment care, might influence the degree of inflammation that develops following a laser dermatological treatment. Due to their higher energy levels and better tissue penetration, some lasers, such as the carbon dioxide (CO_2) laser, can produce more substantial skin inflammation than other laser types.

Hemp is recently legalized by the Foods and Drugs Administration (FDA) of the Ministry of Public Health in Thailand for cosmetics and medical use. *M. oleifera*, *D. scandens*, *C. lappacea* are widely available in Southeast Asian countries. Making this plant a good source of herbal extract to incorporate in facial cream formulation.

Figure 1 Picture of *M. oleifera* Lam. Leaves

Source: https://pixabay.com/photos/plant-moringa-oleifera-superfood-2307261/

2. *Moringa Oleifera*

M. oleifera Lam. is from the Moringaceae family, which in Thailand is called Marum, while in other countries it is commonly known as either Drumstick-tree leaf, Horseradish-tree leaf, Ben-oil-tree leaf, West-Indian-ben leaf. It is also used as a nutrient and a lactagogue for the women who breast feed their infants. The *M. oleifera* Lam is composed of phenolic acids (e.g., gallic acid), flavonoids, and their glycosides (such as astragalin, kaempferol, and quercetin) nitrile, carbamate, and isothiocarbamate glycosides, as well as carbohydrates, proteins, minerals, which are also present.

All the parts of the *M. oleifera* plant are currently being used for their medical purposes. Table 1 shows the components of the plant and their medical uses.

Table 1 Plant parts and its medical uses

Plant Part	Medical Uses
Root	Constipation, rheumatism, inflammations, articular pains, lower back or kidney pain, antilithic, rubefacient, vesicant, carminative, antifertility, anti-inflammatory, stimulating in paralytic diseases; serve as a cardiac/circulatory tonic (Sastri, 1962; Padmarao et al., 1996; Dahot et al., 1988; Ruckmani, 1998)
Leaves	Used as a purgative, as a poultice for sores, as a headache remedy, and as a treatment for piles, fevers, sore throats, bronchitis, eye and ear infections, as well as for scurvy and catarrh; leaf juice is also applied topically to reduce glandular swelling and is thought to regulate blood sugar levels (Morton et al., 1991; Fuglie et al., 2001; Makonnen et al., 1997; Sastri, 1962; Dahot et al., 1988).
Stem Bark	Rubefacients and vesicants are used to treat delirious patients, treat eye illnesses, stop spleen enlargement, stop the development of tuberculous glands in the neck, remove tumors, and heal ulcers. The root bark's juice contains anti-tubercular properties and is applied to the tooth cavity to reduce pain and treat earaches. (Bhatnagar et al., 1961; Shiddhuraju and Becker, 2003)
Flower	High medicinal value as a stimulant, aphrodisiac, abortifacient, and cholagogue; used to treat tumors, enlargement of the spleen, hysteria, and muscle diseases; lowers serum cholesterol, phospholipid, triglyceride, VLDL, and LDL cholesterol to phospholipid ratio; decreases the lipid profile of the liver, heart, and aorta in hypercholesterolemic (Ramachandran et al., 1980; (Bhattacharya et al., 1982; Dahot et al., 1988; Shiddhuraju and Becker, 2003; (Mehta etal., 2003)
Seed	Antihypertensive substances thiocarbamate and isothiocyanate glycosids have been extracted from the acetate phase of the ethanolic extract of Moringa pods, and seed extract exerts its protective effect by reducing liver lipid peroxides. (Faizi et al., 1998; Lalas et al., 2002)

The *M. oleifera* is found in equatorial and sub-equatorial countries, and the tree can grow up to 12 meters tall. The bark of this tree is either smooth or has sunken veinlets, with raised ridges between them. From the main trunk of the tree grows smaller branches that produces many tiny leaves on either side of them, and can reach lengths of 20 to 70 cm (THP, 2021). Phytochemically, *M. Oleifera* leaves have been reported to contain carotene, iron and ascorbic acid and potassium (Makkar et al., 1996) and are an abundant source of essential amino acids such as methionine, cystine, tryptophan, and lysine with a significant number of proteins (Siddhuraju etal., 2003; Makkar et al., 1997). Decoctions and extracts of the plant parts of the *M. Oleifera* are acknowledged for their uses in traditional medicines. For example, in many parts of the Philippines, women who want to increase their production of breast milk include *M. Oleifera* leaves in their food (Anwar et al., 2007; Verma et al., 2009). It has been demonstrated that the moringa leaves are rich in nutrients and can be used in food, and some of its extracts, particularly those that include dietary polyphenols, may be used as "nutraceuticals" to enhance the health of the general populace (Siddhuraju et al., 2003). A significant flavonoid that is found in the *M. oleifera* leaves is called astragalin, which is one of the bioactive components that contains anti-inflammatory properties (Han et al., 2004; Li et al., 2014; Soromou, 2012).

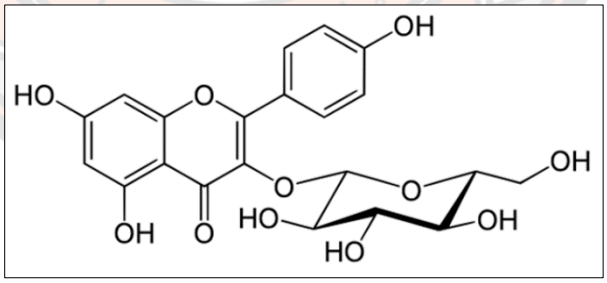

Figure 2 Structure of Astragalin

Source: https://en.wikipedia.org/wiki/Astragalin

Figure 3 Picture of D. Scandens stem

3. *Derris Scandens*

Derris scandens (Roxb.) Benth from the family of Leguminosae, is commonly known in Thai as Thao-Wan-Priang, Brachypteri scandenidis caulis or Hog creeper vine. There are 40 species of the *D. Scandens* that have been categorized as containing analgesic and anti-inflammatory properties, which can be found in most equatorial countries. These can grow as a large woody scandent, or a climbing shrub that can extend up to 30m tall (Muanwongyathi et al., 1981; Bureau of Drug and Narcotic, 2021; Tiangburanatham, 1996). It is a source of biologically active substances that have been used as active ingredients in Thai traditional medicine for pain treatment. The dried stem powder and the ethanolic extract are also recommended as herbal medicines in the Thailand National List of Essential Medicines (NLEMs) for musculoskeletal pain treatment (Ayameang et al., 2020; Health., 2013; Research., 2003). Previous studies reported that benzil derivatives, coumarin, flavone, isoflavone, isoflavone glycosides, terpenes, triterpenes, pterocarpans, and steroids, are phytochemicals and chemical isolates that are found in various parts of the *D. Scandens* (Mahabusarakam et al., 2004; Hematulin et al., 2014;). Traditionally, *D. scandens* has been used in treating muscle pains, (Ayameang et al., 2020; Health., 2013; Research., 2003) and as an anti-tussive, diuretic, expectorant and anti-dysentery agent (Hussain et al., 2015; Kuljittichanok et al., 2018; Hematulin et al., 2014). A recent study signified that Lupalbigenin is one of the components found in 95% ethanolic extract of the *D. Scandens* stem (Chaichamnong

et al., 2018). A research was recently done to an LPS-induced cell changes in Raw 264.7 macrophages were used to examine the inflammatory activity of Lupalbigenin from *D. scandens* aqueous ethanol extract through molecular signaling pathways. The extract of the dried stem of the D. scandens was obtained by using the aqueous ethanol extraction method, which was then used to purify Lupalbigenin. Tumor necrosis factor-alpha (TNF-alpha), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and nuclear factor kappa B (NF-κB) were all successfully suppressed by Lupalbigenin at 1.25 and 2.5 mM. Lupalbigenin also reduced the expression of p38 and JNK in the mitogen-activated protein kinase (MAPK) pathway, which inhibited LPS-induced inflammation (Sriklung et al., 2022).

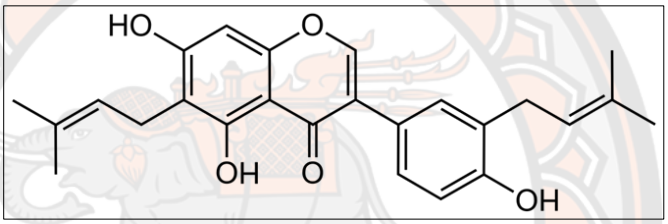

Figure 4 Structure of Lupalbigenin

Source: https://upload.wikimedia.org/wikipedia/commons/8/8f/Lupalbigenin.svg

Figure 5 Picture of *C. Lappacea* leaves

Source: https://www.keycareshop.com/?p=727

4. *Centhotheca Lappacea*

The Poaceae family includes the perennial tufted grass *C. lappacea* (Norsaengsri et al., 2008), commonly known in Thailand as ya hee yum or repair grass (Minale et al., 2021). This plant is commonly found in rain and vine forests, swamps, and wetlands that are adjacent to rivers and streams. It produces stoloniferous runners and rooting at the nodes and grows in clumps and its leaf blades can grow to be 15-30mm long (Kamoltham et al., 2017).

C. lappacea has been used in postpartum care for women for decades. The leaves were dried and burnt in a pot, then the smoke was used to fumigate the vaginal area which helped to heal the lacerations on the labia, and at the same time aided the tightening of the muscles. Consuming an infusion made from boiling the *C. lappacea* leaves in water has been used to nourish the body for many years (McClatchey et al., 1996; Pitiporn, 2014). Ethnopharmacological studies have identified the presence of phytosterols, flavonoids which contain anti-inflammatory and antioxidant activities. Fatty acids and phenolic compounds were found in the leaves of the *C. lappacea* (Kamoltham et al., 2018) and another researcher who used the column chromatography method, isolated 4-coumaric acid and 5,7,4'-trimethoxyflavone from its leaves (Lodovici et al., 2009). A previous study found that the *C. lappacea* had a radioprotective effect on the EA.hy926 cells, with zero evidence of toxicity (Meethang, 2020). Recent investigations sought to determine how

pretreatment with 4-coumaric acid affected the DNA oxidative stress that was brought on by intestinal inflammation in animals. 4-CA can be used to reduce mild intestinal inflammation brought on by Dextran sodium sulfate (DSS), and its effects is linked to the inhibition of the production and activities of COX-2.

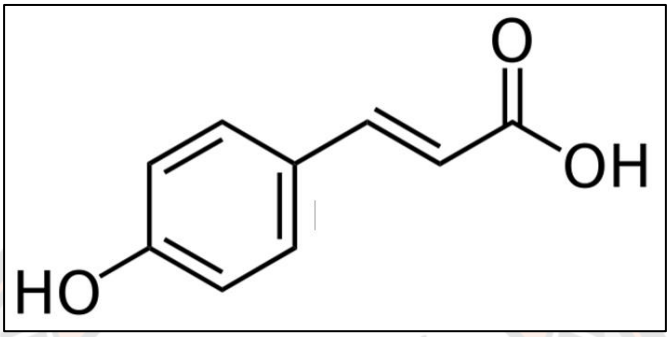

Figure 6 Structure of 4-coumaric acid

Source: https://commons.wikimedia.org/wiki/File:Coumaric_acid_acsv.svg

Figure 7 Picture of Hemp seeds

5. Hemp

Hemp (cannabis sativa) is an angiosperm, which means it has a flower that produces seeds enclosed within a carpel or pistil, which is the plant's reproductive organ. It can be cultivated in a desert or on the top of the coldest mountain peak, according to a cultivation guide (Crini et al., 2020) and prefers a pH balance of 6-7, well-drained land, and aerated loamy soil. Once it reaches its maximum height of 10-15 feet, a hemp crop can be harvested (Martinez *et al.*, 2020). A large and growing body of literature has been published on the phytochemical and chemical constituents of hemp seed. The seed itself contains zinc, vitamins E, C, B1, B2, B3 and B6, and it contains 25% protein, 30% carbohydrates, 15% insoluble fiber, carotene, phosphorus, potassium, magnesium, sulphur, calcium, iron, and zinc (Borhade *et al.*, 2013). Essential oils from the flowers, leaves, wood, fruit, and roots of hemp cultivars demonstrate a good inhibitory anti-microbial growth effect (Stefanini *et al.*, 2006). In recent years, interest in hemp has been largely focused on the seeds, which are nutritionally beneficial for both humans and animals. Recent research has shown that linoleic acid from *A. brasiliensis* decreases NO production and prevents the expression of pro-inflammatory cytokines including TNF-α, IL-6, IL-1, and NOS2 in RAW 264.7 cells. This implies that linoleic acid from *A. brasiliensis* can reduce NO

production and inflammatory activity in RAW 264.7 cells via inhibiting p50 and activating PPARα (Saiki, et al., 2017).

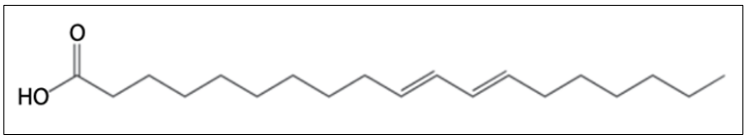

Figure 8 Structure of Linoleic Acid

Source: (Baishya et al., 2017)

6. Ultraviolet (UV) radiation inducing skin inflammation

The sun's energy is the main source of ultraviolet radiation, much of which is absorbed by the ozone layer in the upper atmosphere. The ultraviolet radiation spectrum is divided into UVC (wavelengths 200–280 nm), UVB (wavelengths 280–320 nm), and UVA (wavelengths 320–400 nm), which are categories based on wavelength and energy. Human skin health, including photoaging and photocarcinogenesis, can be negatively and seriously affected by UVA and UVB radiation. When ultraviolet radiation is exposed to human skin, it causes a variety of changes, including the generation of cytokines and pro-inflammatory molecules (Köck et al., 1990; Takashima et al., 1996). Particularly in the epidermis, keratinocytes are the main source of cytokine secretion following UV exposure. The skin's immune system depends heavily on cytokines. Pro-inflammatory mediators such as cyclooxygenase-2 (COX-2) and prostaglandin (PG) cytokine, as well as inflammatory cytokines such as interleukin IL-1 and TNF-α, can be produced by exposure of skin to UV radiation (Rangwala, et al., 2011). The inflammatory systems play a crucial role in the growth of tumors and can cause skin cancer. According to the study, keratinocytes exposed to UVB radiation produce inflammatory cytokines such as IL-lα and IL-1β (Barr et al., 1999). Inflammatory cytokines like TNF-α and pro-inflammatory substances like prostaglandins can be expressed by mast cells because of IL-1α in human skin, which can worsen inflammation in UV-exposed skin (Damian, et al., 2008; Clydesdale, et al., 2001; Barr, et al., 1999;). TNF-α expression from UVB-irradiated keratinocytes has been demonstrated to be increased by IL-1α,

whereas IL-1ß can increase the expression of matrix metalloprotease in human skin via activating NF-κB and Activator protein-1 (AP-1) in the skin (Bashir et al., 2009). MMP exposure to UVB radiation is now better understood and the molecular mechanisms underlying their induction are starting to become clear. According to the Fisher model (Fisher et al., 2002), UV radiation produces ROS and activates growth factors and cytokines through their receptors on the surface of keratinocytes and fibroblasts, which then causes signal transduction through a protein kinase cascade and the nuclear activation of AP-1. The dermis and epidermis are consequently stimulated to produce MMPs like metalloproteinase (MMP) 1 (collagenase), MMP-3 (stromelysin 1), and MMP9 (gelatinase B). These enzymes cause collagen which is the primary protein in the extracellular matrix to degrade.

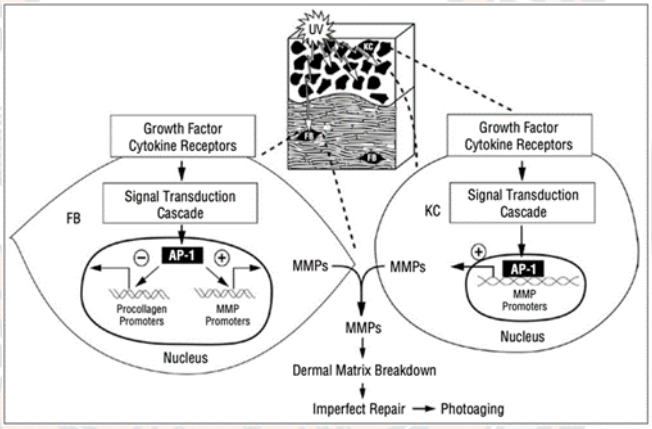

Figure 9 UV-induced signal transduction pathways

Source: Fisher et al., 2022

7. MTT assay for cytotoxic and cell proliferation assay Inflammation process

Screening and safety assessment are the two crucial steps in providing the correct scale of bioactive compounds. Cytotoxicity assays were among the first *in vitro* bioassay methods used to predict the toxicity of substances on various tissues (Berardesca et al., 1995). Researchers have different goals when doing their research. These specific goals have a particular cytotoxicity assay to be used. Four main categories of assays were utilized to observe the reaction of cultivated cells following exposure to potentially toxic substances (Berardesca et al., 1995; Corsini et al., 1995;

(Orjalo et al., 2009; Apte et al., 2006; Ricciotti et al., 2011; Aoki et al., 2012; Chen et al., 2013) cell viability, cell membrane integrity, cell proliferation, and metabolic activity are the measures used when observing the reactions. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction colorimetric assay was used to evaluate the detrimental intracellular effects on metabolic activity. Researchers worldwide used this assay because of its simplicity, popularity and clear description of the intracellular effects.

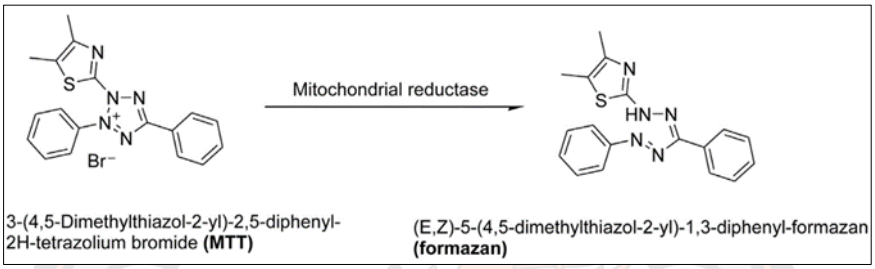

Figure 10 Metabolism of MTT to a formazan salt

Source: Kuete, et al., 2017

In prior research, the depletion of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically functioning cells, MTT was reduced to formazan by NAD(P)H-dependent oxidoreductase enzymes that are in the viable cells. The absorbance was measured at 540 nanometers using a spectrometer and the quantified cells were in a colored solution because the insoluble formazan crystals are dissolved by a solubilizing solution. The greater the metabolical activity and the number of viable cells, the darker the solution will be. Over time, researchers have developed this non-radioactive colorimetric assay system (Mosmann et al., 1983; Bahuguna et al., 2017).

8. Inflammation process

Pain, edema, redness, heat, and loss of function are the most common symptoms of inflammation. Inflammation is classified into acute and chronic forms. Acute inflammation is a non-specific early response to tissue injury that can continue from seconds to days. The major features of the inflammatory reaction are the exudation of build and plasma proteins, as well as the emigration of leukocytes, primarily neutrophils. Chronic inflammation is characterized as a condition in which there is ongoing inflammation over an extended period of time, and it is connected histologically with the presence of lymphocytes and macrophages, blood vessel proliferation, fibrosis, and tissue necrosis. Many factors can influence the pathology and histology of both acute and chronic inflammation.

8.1 Arachidonic acid metabolites

One of the key inflammatory mediators is formed from arachidonic acid metabolism. Arachidonic acid is a 20-carbon (C20) fatty acid that can be obtained directly from food or through conversion from an important fatty acid. It is now present in the cell freely, but it is generally esterified in membrane phospholipids. It is released from membrane phospholipids by mechanical, chemical, and physical stimuli or other mediators activating a cellular phospholipase. Arachidonic acid metabolites, commonly known as eicosanoids, are made by two types of enzymes: COXs and Lipoxygenases (Foegh et al., 1998). Eicosanoids can mediate nearly every stage of inflammation, are detected in inflammatory exudates, and their synthesis is accelerated at an inflammatory site.

8.1.1 Prostaglandin

Prostaglandin E2 (PGE_2) is a bioactive lipid that has been linked to a variety of biological effects related to inflammation and cancer. PGE2 is a member of the prostanoid lipid family, which is a subclass of eicosanoids produced by the oxidation of 20-carbon essential fatty acids (EFAs) that are commonly found in membrane phospholipids. The cellular effects of inflammation are responsible for all clinical manifestations of inflammation, including rubor (redness), tumor (swelling), dolor (pain), and fever. Increased blood flows into the inflamed tissue due to PGE2-mediated vasodilation and increased microvascular permeability cause redness and edema (Ricciotti et al., 2011; Aoki et al., 2012; Chen et al., 2013).

8.2 Cytokines

Inflammatory cytokines are small proteinaceous molecules and are produced by many cell types, especially by macrophage and mast cells. They play an important role in the initiation and maintenance of inflammatory disease as a mediator of cell-cell interaction, their response including activation of endothelium and leukocytes an induction of the acute-phase response.

8.2.1 IL-1α and IL-8

Interleukin-1α (IL-1α) and interleukin-8 (IL-8) expression and release in an *in vitro* reconstructed human epidermis for prediction of *in vivo* skin irritation and/or sensitization (Orjalo et al., 2009; Ricciotti et al., 2011; Apte et al., 2006; Aoki et al., 2012). Immersed keratinocyte cultures were chosen for the development of *in vitro* methods to test irritants or sensitizers because of their ability to produce a wide range of inflammatory mediators (Chen et al., 2013). IL-1 (both alpha and beta forms) is a multifunctional cytokine that regulates inflammatory and immune responses primarily by initiating a signal transduction cascade that eventually induces IL-6 and IL-8 expression (Ricciotti et al., 2011).

8.3 Nitric Oxide (NO)

Nitric oxide (NO) is an important component in physiological processes. NO is both water and lipid soluble, allowing it to move freely within and between cells and serving as a highly efficient local signaling molecule. The roles of NO in vasodilation, inflammation, immunomodulation and oxidative damage to cells and tissues are particularly relevant to the skin (Barker et al., 1991). *In vitro* experiments showed that inflammatory stimuli induce keratinocytes to produce both NO and hydrogen peroxide. These experiments provided the first indication that NO may be an important component of the immune response in human skin. These studies also showed that the epidermal growth factor, which promotes wound healing by stimulating cellular proliferation, is a strong inhibitor of NO synthesis by epidermal keratinocytes. Based on these findings, it has been proposed that NO produced by keratinocytes plays an important role in the regulation of epidermal proliferation during wound healing (Barker et al., 1991).

9. High-Performance Liquid Chromatography (HPLC) analysis

High-Performance Liquid Chromatography (HPLC) is an analytical tool capable of detecting, separating, and quantifying a drug, its various impurities, and drug-related degradants that may form during synthesis or storage. It entails an understanding of the chemistry of the drug substance and aids in the development of analytical methods. HPLC is used not only for quantitative analysis but also for sample fractionation. The purpose of the fractionation is to separate and purify the target compounds in the sample mixture. The mechanism of HPLC has two phases, the mobile phase and the stationary phase. The mobile phase is a liquid that can dissolve the target compound. The stationary phase is a particular part in a column that interacts with the target compound.

The mobile phase is delivered by a pump. The sample is injected into the mobile phase and introduced into a column packed with fine particles that have chemically connected groups such as c18. The elution speed from the column depends on the interaction between the compound and the stationary phase. The target compounds go through the column at different speeds and, during this process, the mixture is separated into each compound. These are eluted from the column and detected by a detector and a plot called a chromatogram is produced based on the signal from the detector. There are two types of elution. As the fundamental HPLC separation method, isocratic mode is widely employed in many laboratories. To elute compounds going through the column, this method employs a fixed ratio of solvent as the mobile phase and offers a stable baseline as well as an unchanging response factor, making it appropriate for the analysis of basic compounds. The disadvantage of this mode is that it is unable to elute complicated substances since they have a wide range of polarities, which could prolong analysis time. By altering the composition and ratio of the mobile phase during system operation, gradient elution is a modified method that facilitates the separation of complicated compounds. Between sample analyses, the mobile phase's elution strength can be changed. As a result, compared to the isocratic mode, the gradient mode is anticipated to offer a shorter time analysis and better resolution. It is appropriate for complicated substances or several analyses of different polarities since it improves peak resolution and sensitivity. However, compared to the isocratic method, this approach requires more complicated

instrumentation and development. In general, the signal is proportional to the concentration of the targeted compound (Gupta et al., 2012).

10. Formulation Development

10.1 Facial cream preparation

The most common dispersions used in cosmetics manufacturing are emulsions. To ensure the stability of oil droplets dispersed in water (o/w type emulsions) or oil (w/o type emulsions), emulsifiers or surfactants are required (Brummer et al., 2006; Moravkova et al., 2011). Creams are semi-solid emulsions composed of oil and water. **Table 2** shows the following raw ingredients that are commonly used in the production of skin creams.

Table 2 Commonly used raw materials in the production of skin creams

Name	Function(s)
Water(Aqua)	This is used in skin creams as a solvent to dissolve other cream ingredients. Creams are prepared using water that is deionized of any toxins, pollution, microorganisms, etc. Depending on the amount of water used in the formulation, water can also form emulsions (Chauhan et al., 2020).
Oils, fat, waxes	Creams must contain oil, lipids, waxes, and the derivatives that these substances produce. Depending on the role, oils, fats, and waxes serve as thickeners, emulsifiers, perfuming agents, and preservatives, among other things.
Emollients	Function by enhancing the skin's capacity to retain water, coating the skin with oil to stop water loss, and lubricating the skin (Jamshiya et al., 2017).
Humectant	These are crucial, multipurpose components that are present in the majority of skin care products. Organic hydrophobic substances are humectants. These are the substances capable of absorbing or holding onto moisture (Reddy et al., 2012).
Active Ingredient	Plays a crucial part in preserving the skin's and the body's physiological health. The formulation of the creams typically includes but not limited to vitamins A, B, C, and E, are among others.
Preservative	This is important to avoid contamination and microbial modification during production, transportation, storage, and customer use.
Color	Used for esthetics
Perfume	Material that emits odors or scents, including a sweet and pleasant perfume.

Cream preparation has become simpler thanks to additives such as emulsifying agents, and the development of new techniques.

10.2 Stability Testing

The cosmetic formulation will be tested for stability using the protocol recommended by the World Health Organization (2009). Product stability testing is an important factor in formulation development with two main objectives; to select an appropriate formulation during product development, and to ensure product stability is maintained throughout the shelf life of the cosmetic product.

The cream will be stored at 50°C for 30 and 90 days. Physical and chemical properties will then be determined. Previously, stability testing was done under ambient conditions for long periods, but this process is no longer economical because of the time and effort required. Chemical kinetics are now proposed to design experimental conditions for the accelerated testing of product stability and the measurement of the concentration of the active substances at different times.

10.2.1 Accelerated stability test

Because of the relatively short development cycle for cosmetic products, accelerated tests allow for the prediction of stability. A common practice is to conduct periodic post-launch monitoring of retained samples stored at ambient temperatures to support the results obtained from accelerated stability testing. The information gathered can also be used to improve the product and the methodology for accelerated stability testing.

10.2.2 Heat-Cool Cycles

Some deficiencies can be revealed more quickly through testing than by passive storage at a constant temperature. Certain types of products can be subjected to freeze-thaw testing which can detect problems such as suspension problems (a tendency to crystallize or cloud), cream instability.

10.2.3 Microbial Testing

Microbial growth is a common issue for both sterilized and unsterilized formulas, which is why excipients are added for microbial preservation. Improper usage, storage and manufacturing are problems commonly encountered that promote microbial growth. Antimicrobial effectiveness testing is done by inoculating microorganisms separately to the sample product, in elevated concentrations, to trigger contamination.

10.2.4 Challenge test

The specification of the challenge test can be seen in the SCCS Notes of Guidance published by the Scientific Committee on Consumer Safety. According to this publication, all cosmetic products are prone to contamination regardless of proper storage and utilization, and preservation tests should be carried out to identify the risk of infection from using these products. *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Candida albicans* and *Aspergillus niger* should come from official collection strains from states in the EU. To achieve sufficient reproducibility, standardized cultures and inoculum preparation are a necessity. For these tests, the recommendation from the SCCS is to use germs that are known to lead to the spoilage of cosmetic products.

CHAPTER III

RESEARCH AND METHODOLOGY

1. Materials and Equipment

Chemicals and reagents

- Dulbecco's Modified Eagle Medium (DMEM), (Gibco, United States)
- Penicillin-streptomycin (Pen Strep), (Gibco, United States)
- Phosphate buffered saline (PBS), (Gibco, United States)
- 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT), (Gibco, United States)
- Enzyme-linked immunosorbent assay (ELISA) kits, (eBioscience, United States)
- Griess assay kit, (Promega, United States)
- ELISA kit for the human PGE2, (Abcam, United States)
- Trypsin-EDTA (0.25% Trypsin-EDTA), (Gibco, United States)
- All solvents used will be of analytical reagent grade (RCI Labscan, Thailand)

Instruments and equipment

- Incubator CO_2 (Forma series II, Thermo Fisher Scientific Inc., (USA)
- Microplate Spectrophotometer (Multimode detector DTX 880,
- Beckmann, Switzerland)
- Micro refrigerated centrifuge (Kubota 3740, Japan)
- pH meter (Mettler Toledo Model S20-K, GmbH Schwerzenbach, Switzerland
- UV test chamber BS-04 (Opsytec Dr. Groebel, Germany; wavelength 313 nm)

2. Preparation of plant extract

The leaves of *M. oliefera* were collected from Phitsanulok province Thailand, and deposited voucher specimen (collection No. J. Engsuwan002). The *M. oleifera* leaves were dried in a hot air oven at 50°C. The dried leaves were ground into rough powder and passed through a 60mm mesh sieve. The powdered leaves were extracted using 50% ethanol in water. It was macerated and shaken for 48 hr. Afterward, it was filtered with filter paper and the ethanol was evaporated under reduced pressure using a rotary evaporator. The protocol was similar to a study reported by Engsuwan et al., 2021.

Additionally, leaves of *C. lappacea* were collected from Wang Thong Phitsanulok province Thailand. The voucher specimen collection No. (05880). The aerial part of *C. lappacea* and hemp seeds were dried in a hot air oven at 50°C, then ground to powder and passed through a 60 mm mesh sieve. The powder was macerated in 95% ethanol for one day while shaken and then filtered through filter paper and the ethanol was evaporated under reduced pressure using a rotary evaporator. Hemp seeds was purchased from Highland Research and Development Institute (Public Organization) with voucher specimen (5784). The steps in *C. lappacea* leaf extractions were followed in a previous research by Meethang et al., 2020.

The stems of *D. scandens* were collected from the Faculty of Pharmaceutical Sciences garden, Naresuan University, Phitsanulok, Thailand with a voucher specimen (No. 05879) was deposited and kept at the PNU herbarium of the Faculty of Science, Naresuan University. The stem of *D. scandens* was sliced into small pieces, and dried at 50°C. It was grounded with a mixer grinder. The dried powder was macerated in 95% ethanol for 3 days. The extract was filtered and evaporated to dryness under reduced pressure. The procedure was correspondent to a prior study describe by Hematulin et al., 2014.

3. Determination biological activities of *M. oliefera* leaf extract, *C. lappacea* leaf extract, *D. scandens* stem extracts, and Hemp seed extract

The cell culture protocol was reviewed by the Naresuan University Institutional Review Board (IRB no. P1-0006/2566).

3.1 Cell cultures

HaCat cell (Immortalized human keratinocyte cell line) (EP-CL-0090) was purchased from Elabscience (Houston, Texas, USA) Keratinocyte Cells were grown and maintained for 2 to 3 days in DMEM complete medium at pH 7.4, supplemented with 20% fetal bovine serum, and 1% penicillin-streptomycin solution in a humidified atmosphere (5% CO_2, 95% air, 37°C). The cells will be grown under standard conditions until 60 – 80% confluency before subculturing. The cell culture media was removed from the culture flask once the cell confluence reached roughly 80%. The leftover medium was then rinsed twice with 3 ml of sterile PBS (pH 7.2-7.4). The adhesion cells were then detached from the flask using 3 ml of 0.25% trypsin-EDTA solution, which was then incubated for 5 minutes. To inhibit the trypsin activity, the same ratio of complete medium was introduced. The detached cell was transferred to a centrifuge tube and spun for 5 minutes at 1500-3000 rpm. Finally, the cells were counted or seeded for the experiment

3.2 Sample Preparation

Hemp and *D. scandens* extract were solubilized using 100% DMSO. While *M. oleifera* leaves extract, and *C. lappacea* leaves extracts were solubilized with 20% DMSO and 80% DMEM-free serum. The concentration of *M. oleifera* leaves extract, *C. lappacea* leaves extracts and Hemp seeds extracts were prepared using 2-fold serial dilution ranging from 3.9 to 500 µg/ml while for *D. scandens* extract the serial dilution used were ranging from 0.20 to 25 µg/ml. However, because a high concentration of DMSO can harm cells, the final concentration of DMSO should be less than 1% when solubilized in a serum-free medium.

3.3 Cytotoxicity testing

An MTT assay was conducted to assess the effects of *M. oleifera D. scandens, C. lappacea* leaf and hemp seeds extracts on the survival of keratinocytes. The mitochondrial reductase enzymes of living cells can reduce the MTT reagent, which has a yellow color, to insoluble formazan which has a purple color and that

color then reflects the number of viable cells present. The cells will be seeded at a concentration of 2×10^4 cells/well in 96-well plates containing DMEM, FBS and Pen strep in a humidified atmosphere of 5% CO_2 at 37°C until the cells completely adhere to the well plate. The medium will be removed and will be replaced with DMEM free serum medium containing plant extracts at various concentrations for 24 hr. MTT solution will then be added to each well and the plate incubated in a humidified atmosphere with 5% CO_2 at 37°C for 4 hr. The MTT solution will be replaced by DMSO AR grade for 10 min and the absorbance will then be read at 595 nm with a microplate reader. The absorbance of the control group (untreated cells) will be considered as 100% cell viability, and the cell morphology will be observed under an inverted microscope.

$$\%\ \text{Cell viability} = \frac{\text{Optical density}_{595}\text{of treated cell}}{\text{Optical density}_{595}\text{of untreated cell}}\ \text{x}\ 100$$

4. Anti-Inflammatory testing

4.1 Sample preparation

HaCat cells were seeded at a concentration of 5×10^5 cells/well in a 24-well plate containing DMEM complete medium in a humidified atmosphere of 5% CO_2 at 37°C for 24 hr. The cells were washed once with 1 ml of PBS and then covered with a thin layer of PBS. The cells were exposed to UVB using a UV test chamber BS-04 with a dose of 35 mJ/cm^2 for NO, IL-1α, IL-8, and PGE2 assay. After exposure, the PBS was replaced with a DMEM-free serum medium containing *M. oleifera*, *D. scandens*, *C. lappacea*, and hemp seed extracts at various concentrations for 24 hr. The cells will then be incubated in a humidified atmosphere with 5% CO_2 at 37°C for 24 hr. The cell culture medium will be collected and stored until used for further experiments.

4.2 Assay for Nitric Oxide

Nitric oxide (NO) released from HaCat cells was assessed by nitrite quantification using a Griess assay kit following the supplier's protocol. HaCat cells were seeded at a concentration of 5×10^5 cells/well in a 24-well plate containing DMEM complete medium in a humidified atmosphere of 5% CO_2 at 37°C for 24 hr. 5

µg/ml of N(G)-monomethyl L-arginine (L-NMMA) was utilized as a positive control because it is a particular inhibitor of this pathway (Rees et al., 1989). The cells were washed once with 1 ml of PBS and then covered with a thin layer of PBS. The cells were exposed to UVB using a UV test chamber BS-04 with a dose of 35 mJ/cm^2 to induce inflammation. Cell culture medium was collected after 24 hr. The 50 µl of supernatant was added to the well plates, then 50 µl of sulfanilamide solution was added to each well and the plates were incubated for 10 minutes. Afterward, 50 µl of N-1-naphthyl ethylenediamine dihydrochloride solution was added and the plate was incubated for an additional 10 minutes. Samples containing nitrite will have a magenta color that absorbs 543 nm light. A calibration curve using standard nitrite of known concentration was created. The results will be calculated as µM of nitrite (µmol of nitrite / l of cell culture medium).

4.3 IL-1α assay by sandwich ELISA

IL-1α released from HaCat was assessed using ELISA kits following the protocols proposed by the manufacturer. The positive control was 5 µg/ml hydrocortisone, while the negative control was untreated cells. First, the microwells were washed twice with 400 µl of wash buffer. It was tapped dry in an absorbent pad to remove excess wash buffer. Next, 100 µl of standard and sample were added into microwells. Followed by 50 µl of Biotin–Conjugate anti-human IL-1α polyclonal antibody. It was sealed and incubated for at least 2 hr at room temperature. After incubation, microwells were washed again four times with 400 µl of wash buffer. Followed by adding 100 µl of diluted Streptavidin-HRP. It was sealed and incubated again for at least 1 hr at room temperature. The incubated microwells were washed again four times with 400 µl of wash buffer. Next, 100 µl of TMB solution was added to all microwells. It was sealed and incubated again for 10 min. Aluminum foil was used to cover and avoid exposure to light. Lastly, 100 µl of stop solution was added to each microwell. The absorbance was measured at 450 nm using a microtiter plate spectrometer. The amount of IL-1α concentration in culture supernatant from Keratinocyte cells was calculated using IL-1α calibration curve.

4.4 IL-8 assay by sandwich ELISA

IL-8 released from HaCat cells was assessed using ELISA kits following the protocols proposed by the manufacturer. The positive control was 5 µg/ml

hydrocortisone, while the negative control was untreated cells. A 96-well high-binding microplate was covered with capture antibodies (1X) 100 µl, sealed, and stored overnight at 4°C. After that, the plate was washed three times with 300 µl/well with washing buffer (1X). To prevent non-specific portion binding, 200 µl/well of ELISA diluent (1X) was added and incubated at room temperature on a shaker (200 rpm) for 1 hour. Before adding 100 µl/well of IL-8 standard and sample, the plate was washed three times. Following that, the plate was washed five times before adding 100 µl/well of the detection antibody (1X) and incubating on the shaker at room temperature for 2 hr or alternatively stored at 4°C overnight. The plate was rinsed five times before adding 100 µl/well Streptavidin/HRP (1X) and shaken at room temperature. After 30 min, the plate was rinsed seven times, 100 µl/well of TMB was added, and the solution was shaken at room temperature for 15 min while avoiding light. The enzymatic reaction was then rapidly stopped by adding 100 µl/well of stop solution. A micrometer plate spectrophotometer was used to measure absorbance at 450 nm. Using the IL-8 calibration curve, the quantity of IL-8 concentration in the culture supernatant of keratinocyte cells was estimated.

4.5 PGE_2 production assay

PGE_2 released from HaCat cells will be assessed using an ELISA kit following the protocols proposed by the manufacturer. The positive control was 5 µg/ml hydrocortisone, while the negative control was untreated cells. The coated microwells were first washed twice in 400 µl of wash buffer. To eliminate excess wash buffer, it was patted dry in an absorbent pad. Following that, 150 µl of 1X Tris Buffer was added to the non-specific binding (NSB) wells, as well as 100 µl of 1X Tris Buffer to the zero standards (B0) wells. Then 100 µl of standards and samples were added to the respective wells. Each well received 50 µl of PGE_2 AP Tracer. Each well also received 50 µl of PGE_2 Antibody. To mix, tap the plate on the counter. It was sealed and incubated at room temperature for 2 hr on a shaker (200 rpm). Aspirate the solution and wells were washed thoroughly five times with 1X wash buffer. Following that, 200 µl of pNPP solution was added to each well. Finally, 5 µl of AP Tracer was added to total activity (TA) wells. It was sealed and incubated at room temperature for 60 to 90 minutes. A microtiter plate spectrometer was used to

detect absorbance at 450 nm. Using the PGE_2 calibration curve, the quantity of IL-1α concentration in the Keratinocyte cell culture supernatant was estimated.

5. Facial cream preparation

The base cream compositions are shown in Table 3. The ingredients in the oil phase will be melted based on their melting points. The wax with the highest melting point is melted first, and the wax with the lowest melting point is added last. The liquid oil ingredients are then added and combined. Carbopol is dispersed separately in water and neutralized with triethanolamine to form a gel for the aqueous phase. Sodium Polyacrylate and glycerin will be dissolved in water and added to the aqueous phase. The oil phase and aqueous phase will be separately heated to 70-75°C at which temperature the oil phase was poured into the aqueous phase while being constantly homogenized at 2000 rpm. The resulting base cream will be homogenized continuously until it reaches 40°C, and Microcare phc will then be added and homogenized until the base cream was homogeneous.

Table 3 Cream base formulation

	Ingredients			
Phase	Trade Name	INCI Name	Function	%w/w
A	DI Water	Aqua	Solvent	q.s
	Carbopol Ulterez-21	Acrylates/C10-30 Alkyl Acrylate Crosspolymer	Thickener	0.1
	Triethanolamine	Triethanolamine	Neutralizer	0.1
B	DI Water	Aqua	Solvent	30.0
	Glycerin	Glycerin	Humectant	3.0
	Sodium Polyacrylate	Sodium Polyacrylate	Stabilizer	0.3
C	Stearic Acid	Stearic Acid	Thickener	4.0
	Cutina EGMS T	Glceryl Sterate	Emulsifier	2.0
	Emulgade F	Cetearyl Alcohol (and) PEG-400 Castor Oil (and) Sodium Cetearyl Sulfate	Emulsifier	2.0
	Lexumul 561	Glyceryl Sterate, PEG-100 Stearate	Emulsifier	0.3
	Emulium Delta	Cetyl Alcohol (and) Glyceryl Stearate (and) PEG-75 Stearate (and) Ceteth-20 (and) Steareth-20	Emulsifier	0.2
	Mineral Oil	Mineral Oil	Emollient	1.0
D	Microcare PHC	Pheoxythanol (and) Chlorphnesin (and) Glycerin	Preservative	1.0
F	Perfume	Perfume	Perfume	q.s

6. Stability Test

The cosmetic formulation was tested for stability following the protocol recommended by the World Health Organization (2009). The cream will be stored at Room Temperature, 4°C, and 50°C for 30 and 90 days and the physical and chemical properties of the cream will be determined.

6.1 Physical stability

The guidelines for facial care products were followed using the Thai industrial standard (TISI, 2010). The pH, general characteristics or phase separation, color, and smell were evaluated.

6.2 Chemical Stability

The formulated facial cream chemical stability was tested using the HPLC method to determine the percentage of the remaining herbal extracts in the cream.

6.3 High-Performance Liquid Chromatography (HPLC) analysis

The percentage of herbal extracts remaining in facial cream at each time point was determined using an Agilent 1260 HPLC system. Phenomenex Luna C18 columns (150 mm x 4.6 m, 5µm) (Phenomenex, Torrance, USA) were used for HPLC analysis. The mobile phases of analysis were as follows; 0.1% (v/v) Formic Acid in H2O (A) and 0.1% (v/v) Formic Acid in ACN (B) with a gradient elution ratio starting from 15% - 40% (B) for 0 - 10 mins, 10 - 20 mins 40% (B), 20-25 mins 40% - 100% (B), 25 - 30 mins 100%. The column temperature was maintained at 40°C, with a constant flow rate of 0.7 ml/min for 30 mins. The injection volume was 10 µl for determining p-coumaric acid content with absorbance evaluated at 310 nm. The method was reported by Minale et al. 2021 with some modifications.

The linoleic acid content in the facial cream was determined using ACN (A) and 0.1% (v/v) Glacial acetic acid (B) (95:5) with an injection volume of 20 µl and was followed at 1 ml/min. The column temperature was not controlled, and the absorbance was evaluated at 205 nm. This method was reported by Kerdsiri et al, 2020. Astragalin was determined on a gradient solvent using 1% (v/v) glacial acetic acid (A) and methanol (B) as the mobile phase. The ratio was as follows 80:20 (A:B) in 3 mins, 80:20 to 65:35 (A:B) in 11 mins held for 14 mins, 63:35 to 0:100 (A:B) in 25 mins held in 5 mins. The contents of coumaric acid, linoleic acid, and astragalin were calculated from the peak area of standard coumaric acid, linoleic acid, and astragalin.

7. Challenge test

The challenge test was carried out to observe microbial contamination in cosmetics during use, storage, or manufacturing by adhering to the United States Pharmacopoeia standard (USP 35, chapter 51) on the topic of antimicrobial effectiveness testing, which used 5 microorganisms (3 bacteria and 2 fungi) for challenge testing.

The test will be conducted using the test organisms *Candida albicans* (ATCC No. 10231), *Aspergillus niger* (ATCC No.16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538). These bacterial and fungal types are known as human pathogens. They were collected from Thailand's Ministry of Public Health's Department of Medical Sciences. The inoculum was made by direct colony suspension and contained 10^8 CFU/ml. In the case of *A. niger*, spores were counted to yield a concentration of around 10^6 spores/ml.

The formulated cream and 0.85% NSS were split into 5 bottles of 10 ml each. For each bottle, 0.85% NSS was utilized as a control. Each bottle received 100 µl of inoculum. Each bottle of NSS was 10-fold diluted three times to 10^{-1}, 10^{-2}, and 10^{-3} at the start. Each 100 µl dilution was placed on an agar plate. The bacteria culture media will be trypticase soy agar (TSA), and the yeast and mold culture medium will be sabouraud dextrose agar (SDA). Following that, the spread plate approach was utilized. *E. coli*, *P. aeruginosa*, and *S. aureus* were cultured for 24-48 hr at 37°C. *C. albicans* and *A. niger* were incubated for 48-72 hr at 25°C. After 7, 14, and 28 days, the leftover bottles of face cream were determined. At the time of testing, 1 ml of mouthwash from each container was mixed with 9 ml of neutralizing diluent (10^{-1}) containing 5% tween 80 and 0.5% lecithin, and then 1-fold diluted to 10^{-2} and 10^{-3}. Furthermore, 100 µl of diluted facial cream was put on an appropriate agar plate and smeared across it. The developing colonies were enumerated, CFU/ml computed, and log CFU/ml calculated (Giorgio et al, 2018; Siegert et al, 2012).

The data were evaluated using the category 2 product criteria. Bacteria had decreased by less than 2.0 logs from the original count after 14 days. There was no growth in yeast and mold counts on days 7, 14, and 28 compared to the first count. (Chapter, 2014; Sutton et al., 2002).

8. Statistical Analysis

Statistical analysis using mean values and standard variance ranges (mean ± SD) will be undertaken, and Student's t-test will be used for comparison between the tested group and the control group and statistical significance will be considered at $p<0.05$ where appropriate. These statistics will be published in Chapter IV below.

CHAPTER IV

RESULT AND DISCUSSION

The study's findings are presented in this chapter. Cytotoxicity testing, anti-inflammatory testing, stability testing, HPLC analysis, and challenge testing are all included.

1. Plant Extract

The plants' leaves, stem, and seeds were extracted with different concentrations of ethanol. The appearance and percentage yield of the extracts is shown in Table 4.

Table 4 Appearances and percentage yields of extracts obtained from leaves stems and seeds with various plants

Type of extract	Appearance	% yield
M. oleifera leaf extract	Dark green sticky mass	12.37
D. scandens stem extract	Dark brown sticky mass	7.79
C. lappacea leaf extract	Dark green sticky mass	4.40
Hemp seed extract	Dark brown sticky mass	8.77

The yield percentages shown in Table 4 are calculated as:

Yield percent = [dry weight of extract after extraction/weight of ground powder extract used before extraction] x 100.

In general, the yield obtained from 50% aqueous ethanolic extract was higher than those from the 95% ethanolic extract. The extracts from *M. oleifera* gave a higher yield (12.37%) than the yields from the 95% ethanolic extracts. The yield obtained from the *D. scandens* stem extracts was 7.79% and from the hemp seed extracts, 8.77%, and the extract obtained from *C. lappacea* gave the lowest yield of 4.74%.

2. Biological Activity testing

2.1 Cytotoxic and cell proliferation testing

To test the abilities of *M. oleifera* leaf extract, *C. lappacea* leaf extract, and Hemp seeds extract against cell viability, HaCat cells were exposed to concentrations ranging from 3.91 to 500 µg/ml for *M. oleifera* leaf extract, *C. Lappacea* leaf extract, and hemp seeds extract, while 0.20 to 25 µg/ml for *D. scandens* stem extract for 24 hr.

HaCat cells were also exposed to 0.20 to 12.5 µg/ml concentrations of *D. scandens* stem extract for 24 hr and no cytotoxicity was observed. However, at concentrations greater than 12.5 µg/ml the viability of the HaCat cells was significantly reduced. ($p < 0.05$). Subsequently, the *D. scandens* stem extract at concentrations of 1 and 10 µg/ml was chosen for further investigation. *M. oleifera* leaf extract, *C. lappacea* leaf extract, and hemp seed extract at concentrations of 1, 10, and 100 µg/ml, were chosen for further investigation.

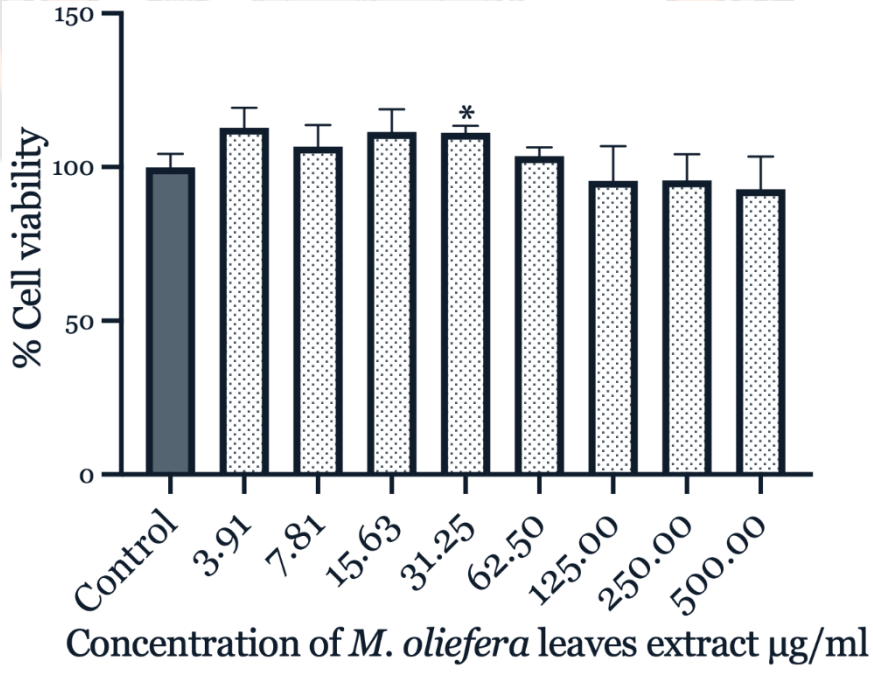

Figure 11 Viability of HaCat cells after treatment of *M. oliefera* leaf extracts

NOTE: The data are presented as mean ± SD (n=3, triplicate) *significantly different versus the control group ($p < 0.05$, t-test)

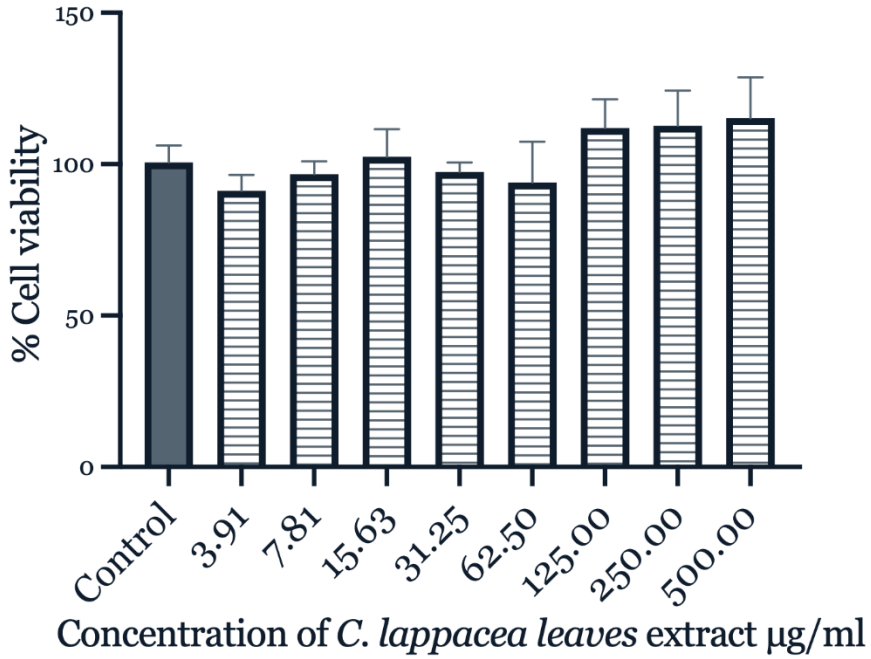

Figure 12 Viability of HaCat cells after treatment of *C. lappacea* leaf extract

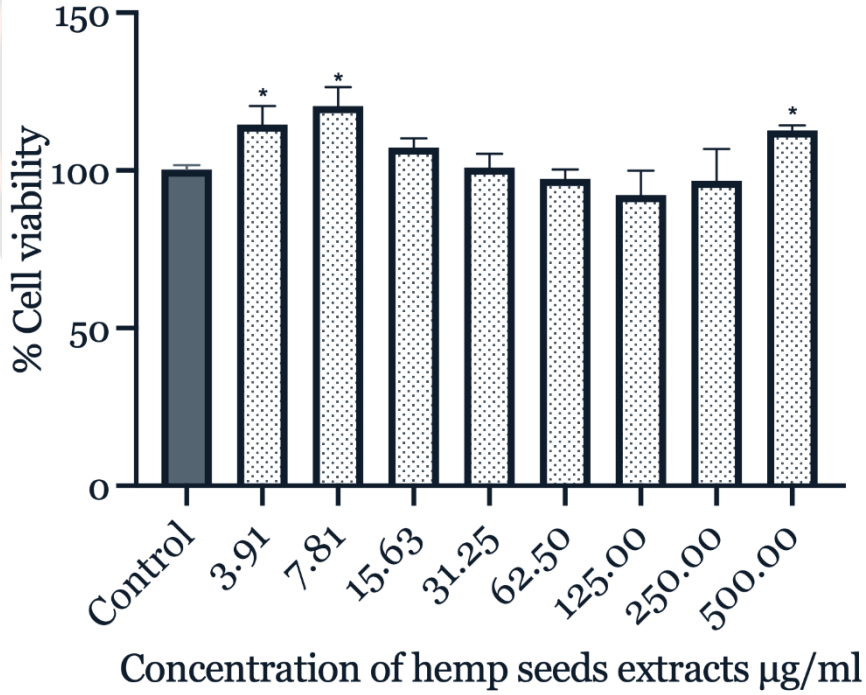

Figure 13 Viability of HaCat cells after treatment of hemp seeds extract

NOTE: The data are presented as mean ± SD (n=3, triplicate) *significantly different versus the control group ($p < 0.05$, t-test).

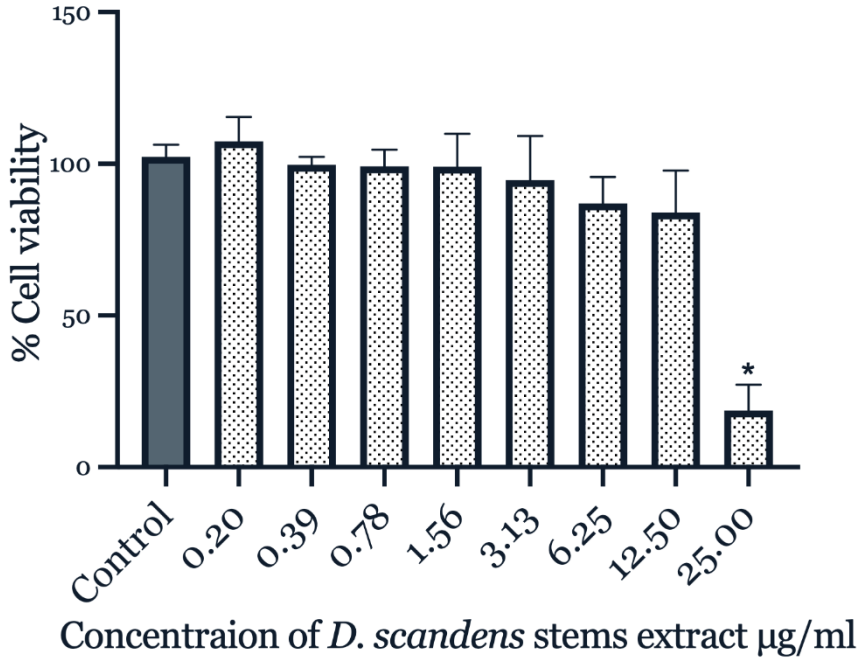

Figure 14 Viability of HaCat cells after treatment of *D. scandens* stem extract

NOTE: The data are presented as mean ± SD (n=3, triplicate) *significantly different versus the control group ($p < 0.05$, t-test).

2.2 Anti-Inflammatory Testing

The DNA of epithelial cells is damaged because of the inflammation resulting from radiation, when the macrophages, fibroblasts, endothelial and epithelial cells, all produce reactive oxygen species (ROS). In reaction to the inflammation, the damaged cells further release pro-inflammatory cytokines and chemokines like Nitric Oxide (NO), IL-1, IL-8, and PGE2. It also appears that the cytokine interleukin-1 (IL-1) can stimulate the expression of the enzyme COX-2 and promote the production of PGE2 in HaCaT cells. This is mentioned in previous studies which stated that IL-1 can also stimulate the expression of COX-2 and SCF in HaCaT cells (Yang et al., 2022).

After the onset of inflammation in the HaCat cells, the effectiveness of *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seed extract against Nitric Oxide (NO), IL-1α, IL-8, and PGE2 was determined.

2.2.1 Nitric Oxide suppression testing on UVB-induced HaCat cells

The effectiveness of *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seed extract against Nitric Oxide (NO), IL-1α, IL-8, and PGE2 in suppressing inflammation in the HaCat cells were also determined in this study.

A Griess kit was used to measure NO production in the culture supernatants. The NO suppression testing process started with cell inflammation of the HaCat cells being induced by exposure to UVB light using a UV test chamber BS-04 source (Opsytec Dr Groebel, Germany; wavelength 313 nm) with a dose rate of 35 mJ/cm^2. HaCat cells were exposed for 22 seconds. The Distance of the cell culture and the lamps were approximately 24cm.

The HaCat cells were then treated with different concentrations of the sample extracts for 24 hr. Figure 15 shows the UVB radiation-induced NO production in the HaCat cells compared to the control group of untreated cells. The *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seed extract significantly decreased the NO production at concentrations of 1, 10, and 100 µg/ml ($p < 0.05$). There was a significant difference in the inhibition activity at each concentration of *M. oleifera* leaf extract. At the concentrations of 10 and 100 µg/ml, the NO level reduced with each higher level of concentration. However, at a lower concentration of 1 µg/ml concentration, the level of NO inhibition was lower than in other concentrations. For *M. oleifera* at concentrations of 10 and 100 µg/ml, there was no statistical significance between the two concentrations. Whereas, at a concentration of 1 µg/ml there was a significant difference between the concentrations of 10 and 100 µg/ml.

C. lappacea leaf extract, on the other hand, inhibited NO production in the HaCat cells in a concentration-dependent manner and significantly increased the suppressive activity of *C. lappacea* leaf extract at 10 µg/ml. NO levels in the HaCat cells at the concentration of 1 and 100 µg/ml have no statistical difference compared to 10 µg/ml concentration. Hemp seed extract at 1 µg/ml concentration shows the highest NO inhibition compared to the other two hemp seed extracts at concentrations of 10 and 100 µg/ml. Furthermore, there was a statistical difference between 1, 10, and 100 µg/ml concentrations of hemp seed extracts.

Moreover, the results for *D. scandens* stem extract at 1 and 10 µg/ml showed significant inhibition activity ($p < 0.05$). However, there is no statistical difference between the two concentrations. The MTT assay results of *C. lappacea* leaf extract indicated that at all concentrations from 3.91 to 500 µg/ml increased cell viability. This can indicate the increase of the NO inhibitory effect of *C. lappacea* leaf extract at a concentration of 10µg/ml. Another possibility is that some of the variety of compounds in this extract also boosted the inhibitory effect in the HaCat cells.

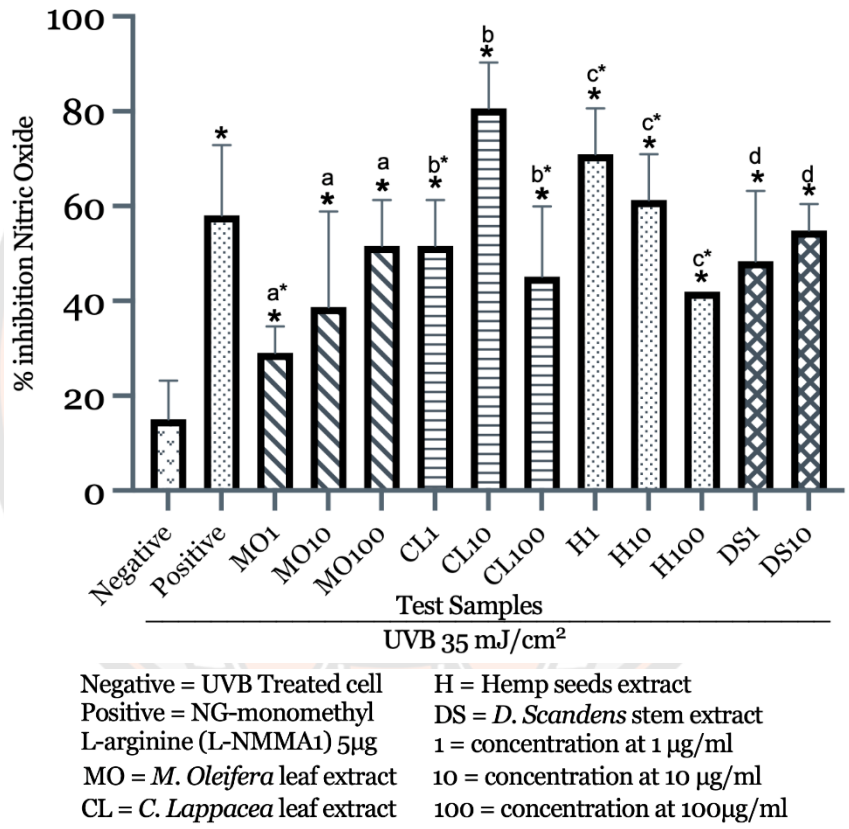

Figure 15 The inhibitory effect on NO of *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seeds extract in UVB-induced inflammatory response in HaCat cells

NOTE: The data are presented as mean ± SD (n=3, triplicate) *$p < 0.05$.

2.2.2 IL-8 testing on UVB-induced HaCat cells using Herbal Extracts

Several studies have investigated the effects of IL-8 in UVB-induced HaCaT cells, and yet the role of IL-8 in UVB responses is unclear. One study found that IL-8 is released in HaCaT cells by UVB irradiation. As well, the same study found that pre-supplementation with certain compounds could reduce this release (Bhadri et al., 2021). Another study looked at the inhibitory effect of IL-6 and IL-8 on UVB-irradiated HaCaT cells and found a suppression of the pro-inflammatory cytokines (Kang et al., 2007).

In the current research, the dosage to induce IL-8 synthesis on HaCat cells was 35 mJ/cm^2. The results demonstrated that the UVB induced the production of IL-8 in HaCat cells. An ELISA kit was used to detect IL-8 production in cell culture media. Hydrocortisone 5µg/ml significantly reduced IL-8 ($p < 0.05$) in UVB-induced cells. The effect was significantly concentration-dependent when the cells were treated with all the plant extracts. The decrease in IL-8 levels in the *M. oleifera leaf* extract-treated group with the concentrations of 1 and 100 µg/ml concentrations of was greater than the decrease observed with the 10 µg/ml concentration. If the concentration-response relationship levels follow a bell-shaped curve, it is possible that when, at 10 µg/ml, the inhibitory effects started to decrease, this caused the cytokine levels to increase, and the inhibitory effects started to peak again at 100 µg/ml. There was no statistical difference from the results of *M. oleifera* leaf extract 1 and 100 µg/ml compared to 10 µg/ml. *C. lappacea* leaf extract at a concentration of 1µg/ml greatly shows a positive inhibitory effect compared to higher concentrations at 10 and 100 µg/ml. There was statistical difference between the result of 1 µg/ml compared to 10 and 100 µg/ml. Hemp seed extract at both lower concentrations of 1 and 10 µg/ml has better inhibitory effects compared to 100 µg/ml. Moreover, there was no statistical difference between 1 and 10 µg/ml compared to 100 µg/ml. Furthermore, *D. scandens* stem extract at both concentrations of 1 and 10 µg/ml displayed an inhibitory effect on the production of IL-8 in the HaCat cells. The result for *D. scandens* stem extract at 10 µg/ml was a statistically significant compared with the result at 1 µg/ml. The above result shows that *C. lappacea* leaf extract at a concentration of 1 µg/ml and *D. scandens* stem extract at 10 µg/ml

concentration have a great inhibitory effect on this test. Given these results, concentrations of 1 µg/ml and 10 µg/ml were used for further investigation.

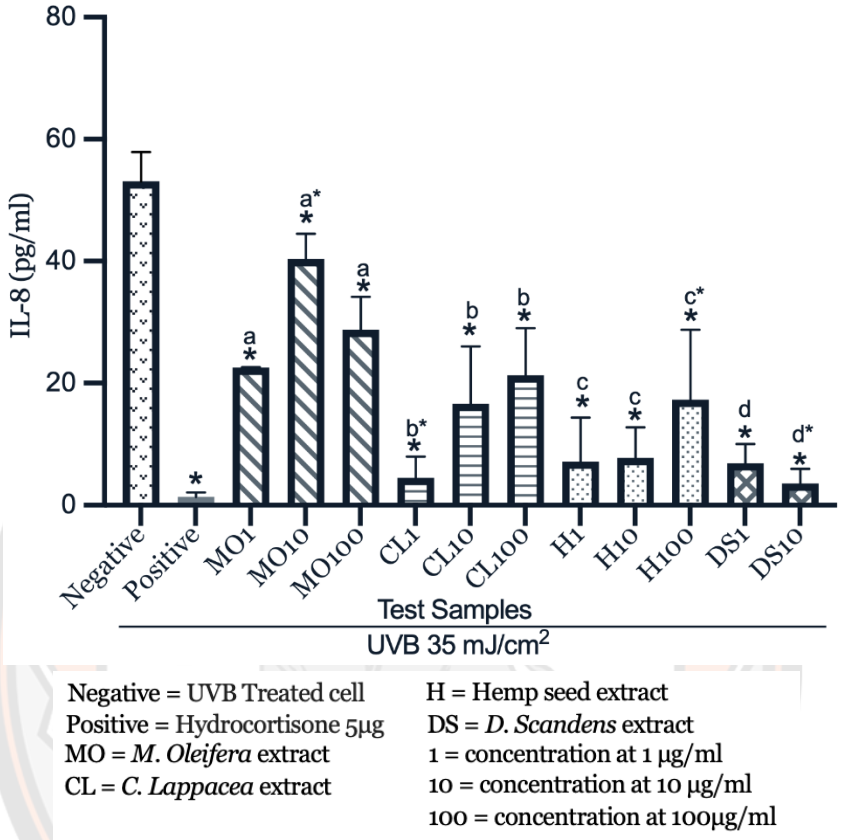

Figure 16 The inhibitory effect on IL-8 of *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and Hemp seeds extract in UVB-induced inflammatory response in HaCat cells

NOTE: The data are presented as mean ± SD (n=3, triplicate) *p < 0.05.

2.2.3 IL-1α testing on UVB-induced HaCat cells using Herbal Extracts

Interleukin-1 alpha (IL-1α) is a cytokine that plays a key role in modulating immune responses and inflammation. In this study, IL-1α levels and the activity of herbal extracts on UVB-induced HaCat cells were tested. The UVB dosage necessary to induce IL-1α synthesis in the HaCat cells was 35 mJ/cm^2. The results for

M. oleifera leaf extract at both concentration of 1 and 10 µg/ml shows great inhibitory effect compared to HaCat cells that were only exposed to UVB without any treatment of plant extracts. Moreover, there was no statistical difference between both results for *M. oleifera* leaf extract. The inhibitory effect of the treatment on the *C. lappacea* leaf extract shows that high concentration demonstrated a positive effect on inhibitory activity. Moreover, there was a statistical difference between the results for both concentrations of *C. lappacea* leaf extract. Hemp seed extract-treated groups were strong with higher concentrations showing a greater effect. However, based on statistical analysis, there is no significant difference between the result for the two concentrations of hemp seed extract. *D. scandens* stem extract treated group showed a clear relationship between concentration and outcome, with a significant increase in the inhibitory effect observed at a lower dose. As well, hydrocortisone 5 µg/ml significantly reduced IL-8 ($p < 0.05$) in the UVB-induced cells. Hydrocortisone is commonly used in scientific studies because it is a well-known and extensively studied compound with a variety of well-established effects, allowing researchers to compare its effects with those of other compounds or treatments against the known and established standards when using hydrocortisone as a positive control, which can help validate and interpret the results. Furthermore, hydrocortisone has unique pharmacological properties that make it useful for researching specific biological systems and pathways.

Of all the herbal extracts tested on IL-1α on the UVB-induced HaCat cells, the largest decrease in IL-1α level ($p < 0.05$) was observed when the cells were treated with the hemp seed extract at a concentration of 10 µg/ml.

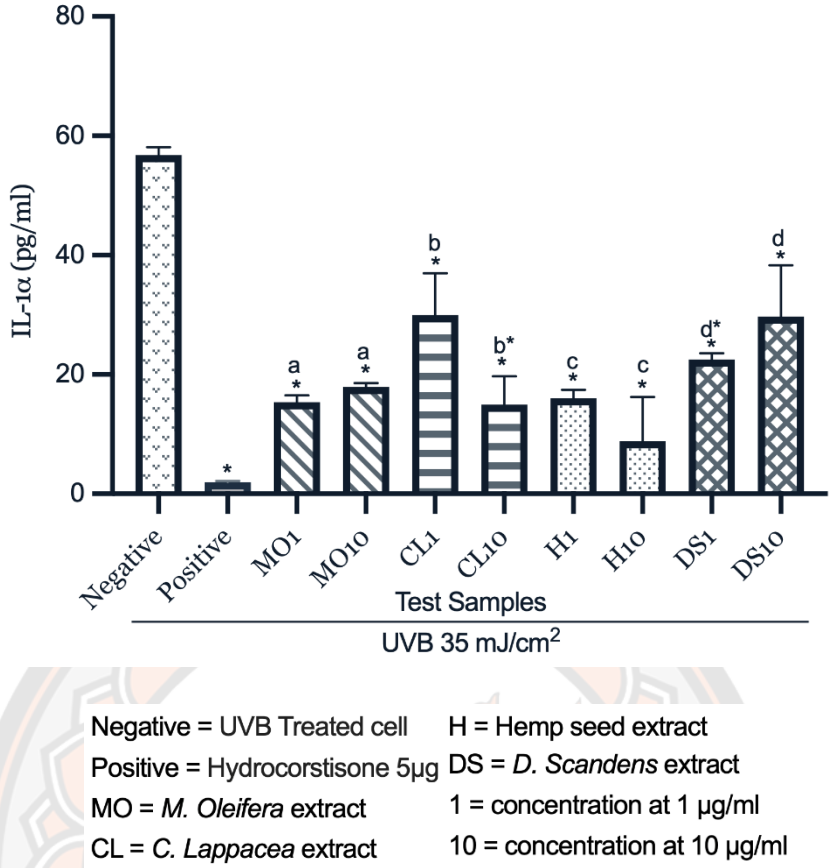

Figure 17 The inhibitory effect on IL-1α of *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seeds extract in UVB-induced inflammatory response in HaCat cells

NOTE: The data are presented as mean ± SD (n=3, triplicate) *p < 0.05**.**

2.2.4 PGE_2 testing on UVB-induced HaCat cells using Herbal Extracts

PGE_2 is induced under various conditions and in various ways. For example, PGE_2 is induced by IL-1α (Yan et al., 2005). As well, PGE_2 can be induced by IL-1 under certain conditions, and by UVB. Pupe et al., (2002) reported that EPA treatment reduces UVB-induced PGE_2 levels in an NHK-conditioned medium, which supports the implication that IL-1 can induce PGE_2 under certain conditions. Additionally, a previous study showed that UVB-irradiation (32 mJ/cm^2) initially induces IL-1 mRNA.

In the current study, a dosage of 35 mJ/cm^2 of UVB was used to induce PGE2 synthesis on HaCat cells. These cells were treated with *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seed extract at concentrations of 1 and 10µg/ml. for 24 hr. An ELISA kit was used to detect IL-8 production in cell culture media.

M. oleifera leaf extract was found to decrease the PGE_2 level in the HaCat cells ($p < 0.05$) (Figure 18). For both concentration 1 and 10 µg/ml, there was no statistical difference on both concertation. Hemp seed extract also were found to decrease the PGE_2 level in the HaCat cells. Moreover, there were no statistical difference between both results. *C. lappacea* leaf extract at concentrations of 1 and 10 µg/ml, the amount of PGE_2 was higher than the amount of PGE_2 achieved by the *M. oleifera* leaf extract and the hemp seed extract at the same concentrations. Alternatively, *D. scandens* stem extract, exhibited a dose activity on the level of PGE_2 at a lower concentration (1 µg/ml) which demonstrated a more positive inhibitory effect than the higher concentrations of the extract 10 µg/ml.

These results suggest that it is possible that the *M. oleifera* leaf extract and hemp seed extract both enhanced the viability of the cells as indicated by the MTT assay results. Another possibility is that some compounds in both extracts may increase the inhibitory activity in HaCat cells.

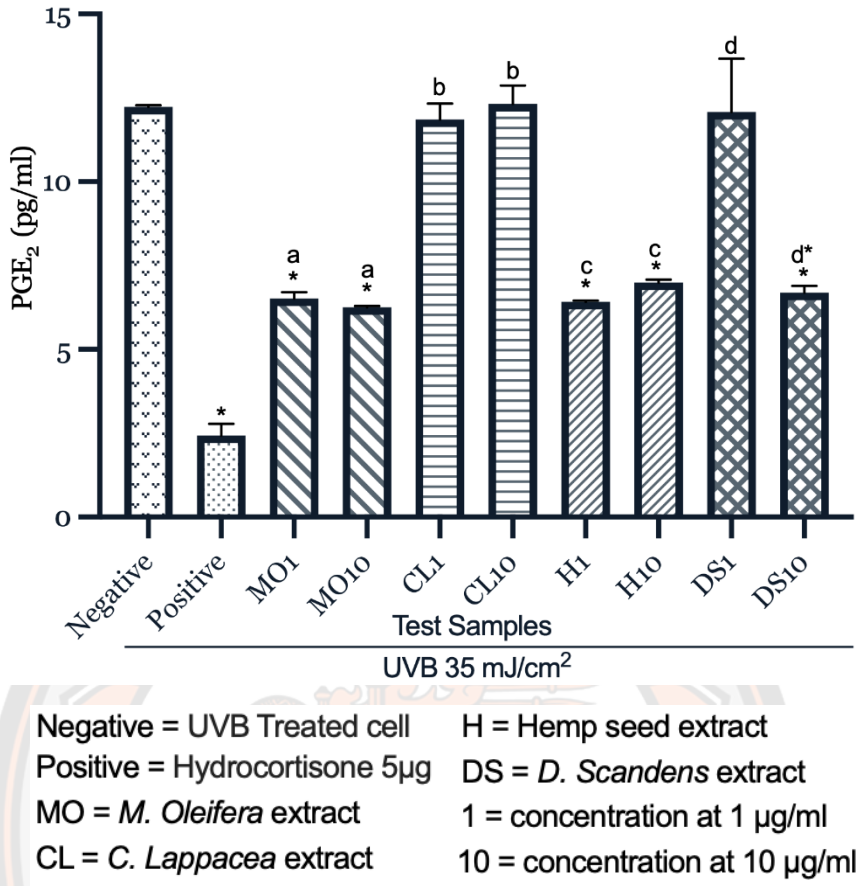

Figure 18 The inhibitory effect on PGE_2 of *M. oleifera* leaf extract, *D. scandens* stem extract, *C. lappacea* leaf extract, and hemp seeds extract in UVB-induced inflammatory response in HaCat cells

NOTE: The data are presented as mean ± SD (n=3, triplicate) *$p < 0.05$.

3. Product Formulation

The facial cream containing herbal extracts was developed to reduce inflammation. The concentration of *M. oleifera*, *C. lappacea*, and hemp seed extract was based on the results of the cytotoxicity assay. The exact location on the body, the skin's health, the existence of any barriers (such as hair or clothing), the characteristics of the substance being absorbed, and the length of exposure are just a few of the variables that can have a significant impact on the absorption value of the skin. It will be based on the absorption value of 0.5 %. Based on our results, *D.*

scandens stem extract at high concentration was toxic to HaCat cells. Therefore, we did not further include *D. scandens* stem extract in the formulation.

4. Study of Facial Cream

When developing skincare products, the stability of facial creams is an important consideration. Stability refers to the ability of the cream to maintain its quality and efficacy over time, which is influenced by factors such as formulation, packaging, and storage conditions. Researchers must carefully design experiments and evaluate factors such as pH, viscosity, and microbiological resistance over time to extend the shelf-life stability of facial creams. This is in addition, of course, to optimize product formulations and packaging, and reducing the risk of skin irritation or other adverse effects. The facial cream being developed in this study was tested for stability using the protocol recommended by the World Health Organization (2009).

4.1 Physical stability of facial cream

A sample of the facial cream was stored at 50°C, 4°C, and another at room temperature. Physical tests are evaluated by checking the color, odor/fragrance, pH value, viscosity, and emulsion stability (signs of separation) of each sample. The testing was done under accelerated conditions. The stability results after 90 days of storage are shown in Table 5-6.

Table 5 Physical stability of facial cream after freeze-thaw cycle testing

Parameters	Time (cycle)	
	0	7
Color	Cream Color 	Cream Color 
Odor	Characteristic odor	Characteristic odor
pH	5.81 ± 0.06	5.91 ± 0.07
Separation	No	No
Viscosity	3120.00 ± 0.80 cp	2122.67 ± 0.32 cp

Table 6 Physical stability of facial cream after accelerated stability testing

Parameters	Time (days)						
	0	30			90		
		RT	4°C	50°C	RT	4°C	50°C
Color	Cream color	Cream color	Cream color	Cream color	Cream color	Cream color	Cream color
							
Odor	Characteristic odor	Characteristic odor	Characteristic odor	Characteristic odor	Characteristic odor	Characteristic odor	Characteristic odor
pH	5.81 ± 0.06	5.84 ± 0.03	5.86 ± 0.01	5.77 ± 0.02	5.85 ± 0.02	5.83 ± 0.03	5.78 ± 0.02
Separation	No	No	No	No	No	No	No
Viscosity	3120.00 ± 80.00cp	3125.67±42.25cp	2668.00±36.47cp	2211.00±107.62cp	3199.67±37.97cp	3287.67±45.83cp	2896.97±19.78cp

*RT – room temperature

The skin has an acidic pH range which under normal conditions is between 4.1 to 6.7 (Proksch et al., 2018). The pH of the developed cream was found to be between 5.77 and 5.86, which was in the normal range for healthy skin. The formulation's color and appearance were observed and confirmed visually. The herbal extracts were distributed uniformly during the face cream formulation. The color of the facial cream was slightly pale, and yellowish-white, due to the presence of plant extracts. The odor was determined to be attractive.

Regarding viscosity, topical formulations with low viscosity have faster spread ability and absorbance, than highly viscous solutions which have an undesirable effect on the skin. The viscosity determinations were carried out using a Brookfield DV-III Rheometer (M13-2100) using spindle number S – CP 52 at 12 rpm. The test was done in triplicate, and the average of three readings was taken. The viscosity was found in the range of 2211.00 – 3199.67 (Dhyani et al., 2019). The overall result in physical stability passed, indicating that the formulation was suitable for skin applications.

4.2 Chemical stability of facial cream evaluated by using HPLC Analysis

The quantitative analysis of astragalin in *M. oleifera* leaf extract, coumaric acid in *C. lappacea* leaf extract, and linoleic acid in hemp seeds extract compared with the standard was determined by HPLC analysis. HPLC method was used to analyze the compound of interest in the extract. The substance which has high compatibility with the mobile phase was eluded first. In this study, linoleic acid was eluted at 7.40 ± 0.12 min. The calculated amount of linoleic acid in 1 mg hemp seed extract was 82.22 ± 0.54 µg/ml following the calculation from linearity of analyzing a series of linoleic acid standards ($y = 33.496x – 1.545$; $R^2 = 0.9998$).

Coumaric acid was eluted at 12.45 ± 0.12 mins. The calculated amount of coumaric acid in *C. lappacea* leaf extract was 33.67 ± 0.09 µg/ml following the calculation from linearity of analyzing a series of coumaric acid standards (y = 100.68 + 14.467; R2 = 1).

Astragalin was eluted at 22.68 ± 0.03 min. The calculated amount of astragalin in *M. oleifera* leaf extract was 84.08 ± 20.01 µg/ml following the calculation from linearity of analyzing a series of astragalin standards (y = 13.425x + 29.381; R2 = 0.9996).

Table 7 % remaining of active compound in facial cream

Parameters	Time (days)						
	0**	30			90		
		RT	4°C	50°C	RT	4°C	50°C
% remaining of active compound in Hemp seed extract	100 ± 0.42	87.25 ± 0.10	94.86 ± 0.08	86.61 ± 0.10	86.42 ± 0.12	93.12 ± 0.56	85.71 ± 0.59
% remaining of active compound in *C. lappacea* leaf extract	100 ± 0.46	92.39 ± 0.23	96.86 ± 0.18	90.21 ± 0.37	90.40 ± 0.12	96.86 ± 0.33	90.16 ± 0.20
% remaining of active compound in *M. oliefera* leaf extract	100 ± 0.42	87.25 ± 0.10	94.86 ± 0.86	86.61 ± 0.47	84.42 ± 0.12	93.12 ± 0.56	85.71 0.59

* RT – room temperature ** adjusted to baseline

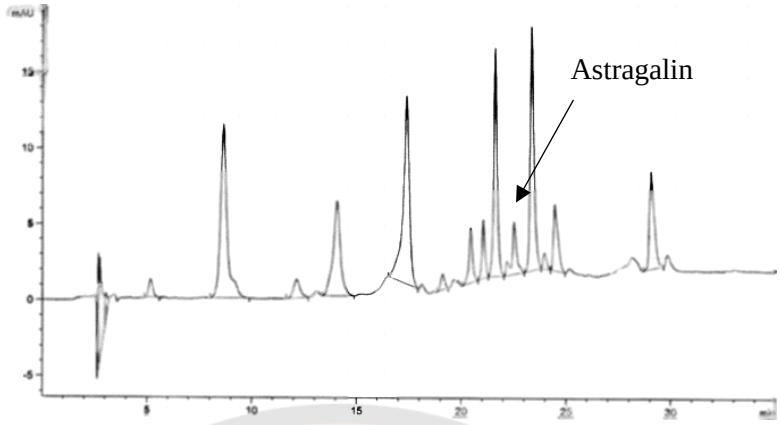

Figure 19 Chromatogram of *M. oleifera* extract 1 mg/ml

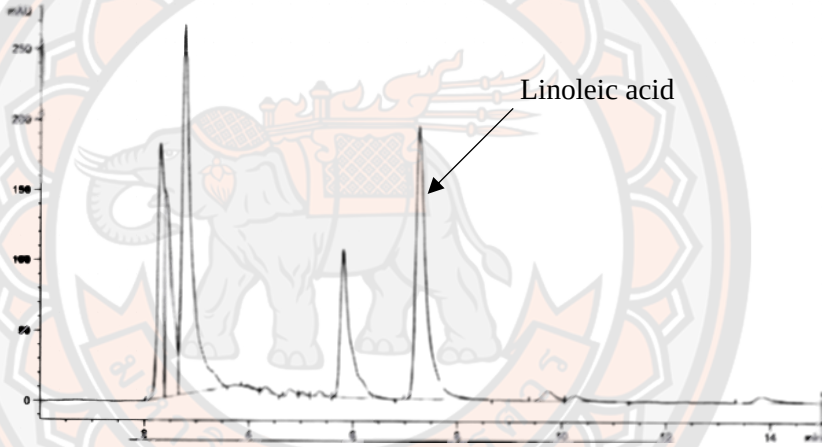

Figure 20 HPLC Chromatogram of Hemp seed extract 1 mg/ml

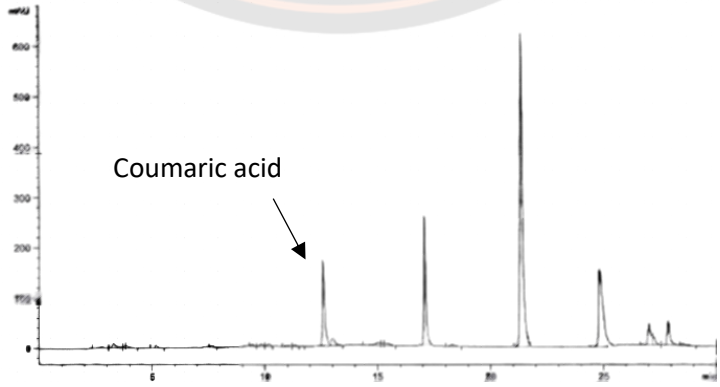

Figure 21 HPLC Chromatogram of *C. lappacea* extract 1 mg/ml

4.3 The efficacy of the preservative in the formulated facial cream containing herbal extracts

Table 8 Product specification

Items	Specifications
Physical test	
Appearance	Cream O/W Emulsion
pH at 25°C	5.81 ± 0.06
Initial Viscosity	3120.00 ± 80.00 cp

The facial cream was tested using the challenge test in the current study described in Chapter III which followed the United States Pharmacopeia standard (USP 35, chapter 51). Microorganisms in cosmetics significantly risk consumer health and high standards of quality monitoring are essential. The major goal of our challenge test was to evaluate the preservative properties of the developed product and detect any microbial contaminations that may occur when being used. the challenge test was effective and appropriate for predicting how the cream would react if contaminated with microorganisms which would cause deterioration and potential toxicity.

Temperature and humidity will cause variations in microbial growth and contamination which is an important consideration in Southeast Asian countries where the weather is normally warm and humid, providing ideal circumstances for such microbial growth. As well, the bacteria being investigated were often found in the environment and when passed by hand or other physical contacts.

Table 9 The log reduction of microorganisms

Microorganism	Log CFU/g				Results
	Initial Count	Day 7	Day 14	Day 28	
S. aureus	5.0	2.2	2.1	2.0	pass
P. aeruginosa	5.1	2.3	2.0	1.6	pass
E. coli	4.9	2.3	2.1	2.0	pass
C. albicans	5.0	2.4	2.1	1.6	pass
A. niger	5.0	2.3	2.1	1.6	pass

The results of the challenge test of the cream showed that this formulation passed the accepted criteria of antimicrobial effectiveness for category 2 products, including topically used products made with aqueous bases or vehicles, nonsterile nasal products, and emulsions including those applied to mucous membranes. The physical and chemical stability of the cream was also tested, and the cream passed those tests. The log reduction of bacterial count from the beginning up to day 14 and day 28 was greater than 2.0 logs. As well, no increase in bacterial count occurred from the 14th day to the 28th day. Additionally, no fungal or yeast increase was detected during the incubation period from day 7 to 28. The results are shown in Table 8. The log reduction of *S. aureus* and *E. coli* at initial count was 5 log reduction and on day 14^{th} it was 2.9 log reduction. On day 28^{th} for *S. aureus* and *E. coli* it was 3 log reduction. *P. aeruginosa* at initial count was 5 log reduction and on day 14^{th} it was 3 log reduction. On day 28^{th} *P. aeruginosa* it was 3.4 log reduction. Lastly, *C. albicans* and *A. niger* at initial count was 5 log reduction and on day 14^{th} it was 2.9 log reduction. On day 28^{th} *C. albicans* and *A. niger* it was 3.4 log reduction. The result shows that if the log reduction is greater than 2 log reduction which means it passed the criteria. Indicating that on day 14^{th} it reduces more than 2 log reduction and on day 28^{th} there were no bacteria, fungi, and yeast increase from initial count. This also signifies that the preservative use was effective.

CHAPTER V

CONCLUSION

In this research, the cytotoxicity and anti-inflammatory properties of *M. oleifera, D. scandens, C. lappacea,* and hemp seed extract were evaluated. As well, a facial cream was developed and its properties such as, color, appearance, and odor were evaluated after stability over time, together with the efficacy of the preservatives used in the facial cream.

A cytotoxicity study was conducted before testing the anti-inflammatory activities of the extracts. *M. oleifera, C. lappacea,* and hemp seed extract extracts significantly improved the viability of the HaCat cells, at all concentrations. The non-cytotoxic concentrations of *D. scandens* stem extract was 0.20 to 12.5 µg/ml. At higher concentration, *D. scandens* stem extract reduced the viability of HaCat after 24 hr. In addition, significant inhibition of NO release by UVB-induced HaCat cells occurred with 10 µg/ml concentrations of *C. lappacea* leaf extract. Moreover, hemp seed extract. *M. oleifera* leaf extract and *D. scandens* stem extract also showed inhibitory effects. The *D. scandens* stem extract at 10 µg/ml, and *C. lappacea* leaf extract at 1 µg/ml significantly inhibited the production of IL-8. Hemp seed extract at 10 µg/ml shows the positive inhibition of IL-1α. The concentrations of 1 and 10 µg/ml of *M. oleifera* leaf extract and hemp seed extract demonstrated a high level of inhibition of PGE_2. However, *C. lappacea* leaf extract at concentrations 1 and 10 µg/ml did not show positive results. Furthermore, *D. scandens* stem extract only shows good inhibitory effect at 10 µg/ml.

The stability of the developed facial cream over 3 months at room temperature, 4°C, and 50°C, was satisfactory, with the chemical stability indicating that the bio-active markers for the extracts were maintained over the 3 months test period. In addition, we have shown that the preservatives used in this facial cream were effective in protecting the facial cream against microbial growth over the long term.

It can be concluded that *M. oleifera, D. scandens, C. lappacea*, and hemp seed extracts can inhibit inflammation of HaCat cells. The cosmetic cream containing these extracts was successfully developed with good stability profile.

However, given the sensitive nature of the use of cosmetic creams, further clinical studies are necessary to further prove these results.

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APPENDIX

APPENDIX A APPROVAL DOCUMENT FOR PROCESSING THIS RESEARCH STUDY FROM GRADUATE SCHOOL OF NARESUAN UNIVERSITY

Announcement

The Graduate School, Naresuan University

Approval of Thesis Proposal to Conduct Research

No. 41/2023

The Graduate School approved **Miss NINO DE MESA,** student ID: 63063934; Master of Science Program in Cosmetic Sciences, to conduct research. The approved thesis proposal entitled: **"DEVELOPMENT OF FACIAL CREAM FOR ANTI-INFLAMMATORY REDUCTION CONTAINING HERBAL EXTRACTS"** with Associate Professor Dr. Neti Waranuch as the thesis advisor.

Announced date: March 15, 2023

Krongkarn Chootip

(Associate Professor Dr. Krongkarn Chootip)
Dean of the Graduate School, Naresuan University

APPENDIX B APPROVAL DOCUMENT FOR HUMAN ETHIC COMMITTEE OF NARESUAN UNIVERSITY

AF 07-09/5.0

COE No. 014/2023
IRB No. P1-0006/2566

NARESUAN UNIVERSITY
INSTITUTIONAL REVIEW BOARD
99 Moo 9 Thapho, Naresuan University, Phitsanulok 65000, Thailand, Tel 66 5596 8752

Certificate of Approval (Initial Submission)

The Institutional Review Board of the Naresuan University, Phitsanulok, Thailand, has approved the following study which is to be carried out in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Study Title : Development for Anti-Inflammatory Reduction containing herbal extracts
Study Center : Naresuan University
Principal Investigator : Miss Nino De Mesa
Review Method : Exemption Review
Continuing Report : No progress reports

Signature: .....
(Somboon Tansuphaswasdikul,MD)
Chairperson, The Institutional Review Board
Naresuan University

Date of Approval : February 20, 2023

Note.

1. No progress report, final report required
2. Any approved exemption protocol modification, require protocol amendment or new protocol submission